ACTIVITY OF ANTIMICROBIAL PEPTIDES AGAINST THE CAUSAL AGENTS OF COMMON SCAB, BLACK LEG AND TUBER SOFT ROT DISEASES OF POTATO

CALUM R. WILSON[1] & A.J. CONNER[2]

[1]Department of Agricultural Science, University of Tasmania, GPO Box 252C, Hobart 7001, Australia. [2]New Zealand Institute for Crop & Food Research Ltd., Private Bag 4704, Christchurch, NewZealand

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ABSTRACT

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Several lytic peptides were assessed for antimicrobial activity against pathogenic isolates of *Streptomyces scabies* and *S. acidiscabies* (causal agents of common scab disease of potato), and *Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *carotovora* (causal agents of black leg and tuber soft rot diseases of potato). No significant phytotoxicity was shown *in vitro* against potato plantlets at concentrations that could be expected to occur within the cells of transgenic plants expressing genes encoding these antimicrobial peptides. Strong inhibition of all *Streptomyces* sp. isolates was observed following incubation with cecropins A, and P1 and magainin 2 amide. The *E. carotovora* isolates were inhibited strongly by these three peptides and also cecropin B. In combined challenges, synergistic activity between cecropin A and magainin 2 was suggested. This is the first report of activity of these antimicrobial agents against an actinomycete.

KEYWORDS: *Streptomyces* - *Erwinia* - cecropin - magainin.

INTRODUCTION

The soilborne bacterial diseases common scab, black leg, and tuber soft rot cause serious economic losses for potato growers and processors worldwide. These may be due to a depreciation of yield, a reduction in the quality of harvested produce, and/or the costs involved in disease management. Current control measures are not always successful and no reliable disease resistance exists in commonly grown commercial cultivars. Traditional potato breeding for bacterial resistance is limited by the availability of suitable genes within sexually compatible organisms. However, genetic manipulation using recombinant DNA and plant transformation greatly expands the potential sources of disease resistance genes (Gasser & Fraley 1989). In addition, genetic engineering allows the introduction of specific traits into commercial cultivars without the inevitable genetic reorganisation associated with sexual hybridisation in traditional breeding. This has the advantage of incorporating disease resistance genes into specific potato cultivars, whilst retaining existing desirable combinations of elite traits.

In recent years, lytic peptides with antimicrobial activity have been identified from various biological sources. Some of the better studied members include the cecropins (originally found in the Ceropia moth; Hultmark *et al.* 1980), defensins (first found in humans, Ganz *et al.* 1985), magainins (from the South African clawed frog, *Xenopus laevis*, Giovannini *et al.* 1987, Zasloff 1987), melittins (from the honeybee, Haberman 1972), thionins (from plants, Garcia-Olmedo *et al.* 1989, Apel *et al.* 1990) and bombinins (from the European and Asian toads, *Bombina* sp.; Gibson *et al.* 1991, Simmaco *et al.* 1991). These small peptides consist of 23-47 amino acids and appear to be major components of an inducible host defence system which acts by disrupting cellular membranes.

Unlike many of the other peptides, the cecropins and magainins do not have significant haemolytic activity and show selectivity for prokaryotic cell

membranes (Boman & Hultmark 1987, Zasloff 1987, Chen *et al.* 1988, Wade *et al.* 1990). Cecropins in particular have been shown to have activity against a wide range of phytopathogenic bacteria (Jaynes *et al.* 1987, Nordeen *et al.* 1992, Hightower *et al.* 1994).

The incorporation of genes encoding such antimicrobial factors into crops like potato may be useful in increasing the level of resistance to important bacterial diseases. This paper describes *in vitro* tests to determine the feasibility of using this approach.

MATERIALS AND METHODS

Bacterial isolates

Pathogenic strains of *Streptomyces scabies* (isolates 20 and 25) and *S. acidiscabies* (27) were isolated from diseased lesions on Russet Burbank potatoes in Tasmania, Australia. An isolate of *S. scabies* (32) was also obtained from the culture collection of the Department of Rural Affairs, Victoria, Australia. All were maintained on glycerol asparagine tryptone agar (Pridham *et al.* 1957) slopes at 4° C. Isolates of pathogenic *Erwinia carotovora* subsp. *atroseptica* (ICMP 8975) and *Erwinia carotovora* subsp. *carotovora* (ICMP 8972) were obtained from the International Collection of Micro-organisms from Plants, Landcare, Auckland, New Zealand. Overnight cultures in nutrient broth were stored in 10% glycerol at -70° C.

Peptides

Six antimicrobial peptides (cecropin A, cecropin B, cecropin P1, magainin 1, magainin 2 and magainin 2 amide) were purchased from Sigma Chemical Co. (USA). Caerin 1.1 (Stone *et al.* 1992) was synthesised using an Applied Systems 431A peptide synthesiser with FastMoc chemistry. Peptides were reconstituted in deionised water to stock concentrations of 0.75 $mg.ml^{-1}$ a.i. and filter sterilised (Millipore membrane pore size 0.2 μm).

Peptide challenges against *in vitro* potato plantlets

Apical shoot tips (2-3 nodes of approximately 1 cm in length) from axenic cultures of potato (*Solanum tuberosum* L. cultivar Iwa) were incubated in 1.5 ml microcentrifuge tubes containing 100 μl of the salts, vitamins and sucrose of MS medium (Murashige & Skoog 1962) adjusted to pH 5.8. This medium was amended with individual peptides at 37.5 $\mu g.ml^{-1}$ a.i., with additional water added to controls. In treatments involving combinations of cecropins and magainins, 37.5 $\mu g.ml^{-1}$ a.i. of both peptides was added.

The plants were incubated at 25° C under 90 $\mu mol.m^{-2}.s^{-1}$ fluorescent light with a 16 hour photoperiod. The total number and length of all roots produced by each shoot tip were recorded after one week.

Peptide challenges against bacterial growth

Inocula of *Streptomyces* isolates were prepared by scraping the surface of a highly sporulating agar plate into 3 ml sterile water. For *Erwinia* isolates, overnight cultures grown in tryptone yeast extract (TYE) broth (Pridham *et al.* 1957) were used. These inocula were diluted 1:20 v/v into sterile TYE broth (pH 7.2) to provide standard inocula for all experiments. Aliquots of 47.5 μl were dispensed aseptically into sterile 1.5 ml microcentrifuge tubes, and amended with 2.5 μl of peptide stock solutions or additional water for controls. Peptide toxicity was tested initially at 37.5 $\mu g.ml^{-1}$ a.i.. In treatments involving combinations of peptides, both were added at 37.5 $\mu g.ml^{-1}$ a.i.. In subsequent experiments to determine the dilution end-point of peptide efficacy, selected peptides were tested from 4.7 to 50 $\mu g.ml^{-1}$ a.i. against single *Erwinia* and *Streptomyces* isolates. Three replicates per treatment were performed for all experiments.

The cultures were incubated at 24° C (*Erwinia*) or 30° C (*Streptomyces*) for 24 hours under stationary conditions. Two subsamples (10 μl each) from each replicate tube were then serially diluted to 10^{-2} and 10^{-4}, and 100 μl from each dilution spread onto either nutrient agar (*Erwinia*) or yeast extract-malt extract agar (*Streptomyces*; Pridham *et al.* 1957). The plates were incubated at 24° C for 3 - 6 days after which the number of colony forming units (cfu) were determined by counting all discrete colonies (for fewer than 3000 colonies/plate) or by counting colonies within a representative sector of the plate and estimating the total. The number of cfu from the duplicated dilution plates for each treatment was averaged to minimise effect of mixing errors.

STATISTICAL ANALYSES

Recorded data was evaluated by analysis of variance using Microsoft Excel 4.0. Experiments showing significant variation between means were assessed further to determine differences between individual treatments using Duncan's Multiple Range test ($P = 0.05$).

RESULTS

NON-PHYTOTOXICITY OF PEPTIDES TO *IN VITRO* POTATO CULTURES

No evidence was found for phytotoxicity of the antimicrobial peptides to potato plantlets (Table 1). For all the peptide treatments, the number and length of roots developing on culture shoot tips of the potato cv. Iwa did not significantly differ from the control. This was observed for both single and combined applications of peptides at the standard concentration of 37.5 μg.ml^{-1} a.i. (Table 1).

TOXICITY OF PEPTIDES TO POTATO PATHOGENS

Cecropin A and magainin 2 amide were most effective against the *Streptomyces* sp. isolates tested (Table 2). Magainin 2 was moderately effective whereas magainin 1,cecropin B and caerin 1.1 had little or no effect. Combined treatments of cecropin A and magainin 2 gave greater inhibition than the individual peptides suggesting some synergistic behaviour. By contrast, the combination of cecropin B and magainin 2 was not more effective than single applications of either peptide.

Cecropin treatments (including cecropin B) showed extreme activity against the *Erwinia* isolates (Table 2). Magainin 2 amide was also highly effective, with magainin 2 having moderate activity, and magainin 1 and caerin 1.1 having minimal or no effect. The extreme activity of cecropin treatments obscured any synergistic activity that may have existed between cecropins with magainin 2.

DETERMINATION OF DILUTION END POINT FOR PEPTIDE EFFICACY

Cecropin A showed significant activity at all tested concentrations against *Streptomyces* isolate 25, the effect diminishing with dilution, whereas the inhibitory action of magainin 2 was ineffective at concentrations below 12.5 μg.ml^{-1} a.i. (Fig. 1a).

Table 1. Effect of antimicrobial peptides on the production and growth of roots of potato (cv. Iwa)

Treatment[A]	Experiment1		Experiment 2	
	root no.[B]	root length (mm)[C]	root no.	root length (mm)
Control (water)	4.5	8.0	5.7	5.9
Magainin 1	4.2	7.7	-	-
Magainin 2	5.4	9.7	6.2	6.4
Magainin 2 amide	4.9	7.1	-	-
Cecropin A	4.2	9.9	6.4	4.7
Cecropin B	4.1	7.2	5.0	4.5
Cecropin P1	-	-	3.9	4.9
Caerin 1.1	-	-	6.1	7.4
Cecropin A + Magainin 2	-	-	6.0	7.1
Cecropin B + Magainin 2	-	-	5.3	5.9
P	n.s.	n.s.	n.s.	n.s.

[A]Shoot tips (2-3 nodes) were incubated at 24° C for 1 week in 100μl MS medium amended with either water or antimicrobial peptide (at 37.5 μg.ml^{-1}).

[B]Figures are mean number of roots produced / shoot tip from ten replicates.

[C]Figures are mean length of root growth / root / shoot tip from ten replicates. (- = not tested; n.s. = not significant (5%))

Table 2. *In vitro* activity of antimicrobial peptides on bacterial pathogens of potato.

Treatment [A] (Expt. 1)	*S. scabies* (isol. 20)	*S. scabies* (isol. 25)	*S. acidiscabies* (isol. 27)	*S. scabies* (isol. 32)	*E. carotovora* (isol. 8972)	*E. carotovora* (isol. 8975)
Control (water)	324.3[B] a[C]	421.5 b	662.3 a	407.0 a	6582.0 a	5449.5 b
Cecropin A	1.0 e (99.7)[D]	19.9 c (95.3)	64.5 d (90.3)	18.3 d (95.5)	0.0 d (100)	0.0 c (100)
Cecropin B	218.9 b (32.5)	429.1 b	471.5 b (28.8)	215.9 b (47.0)	0.0 d (100)	0.3 c (100)
Magainin 1	123.9 c (61.8)	655.8 a	288.8 c (56.4)	171.3 c (57.9)	5775.1 b (12.3)	6474.0 a
Magainin 2	64.8 d (80.0)	88.5 c (79.0)	122.8 d (81.5)	217.1 b (46.7)	3134.8 c (52.4)	6337.5 a
Magainin 2 amide	1.0 e (99.7)	19.6 c (95.3)	63.1 d (90.5)	13.6 d (96.7)	42.8 d (99.4)	43.8 c (99.2)
P	< 0.001	< 0.001	0.004	< 0.001	< 0.001	<0.001
sed[E]	13.2	36.6	45.2	14.2	237.2	119.9
df	18	18	18	18	18	18
(Expt. 2)						
Control (water)			626.2 a	622.7 a		6643.0 a
Caerin 1.1			478.8 b (23.5)	610.2 a		5976.7 b (10.0)
Magainin 2			637.0 a	124.2 c (80.1)		5024.0 c (24.2)
Cecropin A			320.8 c (48.8)	101.0 c (83.8)		0.0 d (100)
Cecropin B			456.5 b (27.1)	328.3 b (47.3)		0.0 d (100)
Cecropin P1			321.2 c (48.7)	76.8 c (87.7)		0.0 d (100)
Cecropin A + Magainin 2			238.2 d (62.0)	49.3 c (92.1)		0.0 d (100)
Cecropin B + Magainin 2			613.8 a	126.5 c (79.7)		0.0 d (100)
P			< 0.001	0.008		<0.001
sed			25.7	26.2		181.1
df			16	16		16

[A] Bacteria were incubated in 50μl volumes of TYE broth amended with either water or 1-2 antimicrobial peptides (at 37.5μg.ml^{-1} a.i. each).

[B] Figures are means from three replicates of counts of colony forming units after 24 hours incubation from the test solutions diluted 10^{-2} (*S. acidiscabies* isol. 27) or 10^{-4} (all other isolates).

[C] Differing letters indicate significant differences between treatment means (Duncan's multiple range test, $P = 0.05$).

[D] Figures in brackets are percentage inhibition [(1 - mean cfu peptide treatment / mean cfu control) x 100]

[E] sed = standard error difference; df = degrees of freedom

Cecropin A and cecropin P1 significantly inhibited the *Erwinia* isolate 8975 at all dilutions, showing total inhibition at concentrations above 12.5 μg.ml^{-1} a.i.. Complete inhibition was observed also for treatment of magainin 2 at 50 μg.ml^{-1} a.i.. The efficacy of magainin 2 reduced with dilution and was insignificant at 6.25 μg.ml^{-1} a.i. (Fig. 1b).

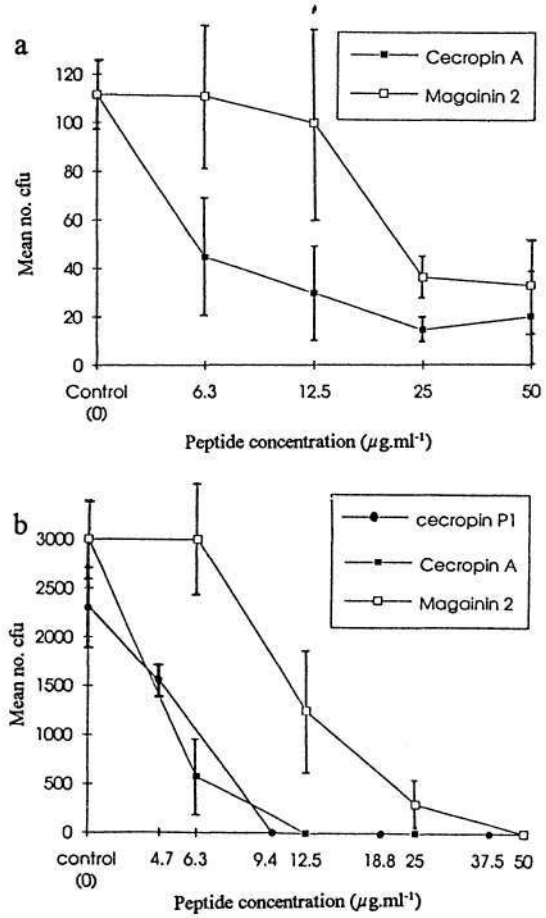

Figure 1. Effect of peptide concentration on *in vitro* inhibition of selected pathogens a) *Streptomyces scabies* (isolate 25). b) *Erwinia carotovora* subsp. *atroseptica* (ICMP 8975). Error bars are 95% confidence limits.

DISCUSSION

The transfer of genes encoding antimicrobial peptides to potato plants has considerable potential for improving resistance to important bacterial diseases. There are two important prerequisites for this approach. The peptides must be non-toxic to potato plants, and show significant inhibitory activity against the target pathogens. In the work reported here we have investigated these issues for seven peptides with known antimicrobial activity and the causal agents of common scab, black leg and tuber soft rot diseases of potato. Based on conservative calculations of transgene expression within plant cells, we estimate that such peptides could be expected to accumulate to about 35-40 μg.ml^{-1} in the cell sap. Therefore, the toxicity of the peptides to cultured potato plants, and pathogenic isolates of *Streptomyces* and *Erwinia*, were evaluated at 37.5 μg.ml^{-1} a.i.

The results demonstrate clearly that certain antimicrobial peptides are highly active against potato pathogens (Table 2) at concentrations non-toxic to potato plants (Table 1), although the effect on potato growth and development of continual exposure to these peptides through expression within transgenic cells may differ to that observed in *in vitro* experiments. Of the peptides tested, the cecropins may have more promise for targeting *Streptomyces* and *Erwinia* infections. In this respect it is noteworthy that cecropins A and P1 have higher activity against *Streptomyces* sp. than cecropin B. Unlike Boman (1991) who reports cecropin P1 as active only against Gram negative bacteria, we found significant inhibition of Gram positive *Streptomyces*. Our results also confirm earlier studies which establish the high sensitivity of plant pathogenic *Erwinia* isolates to cecropin A (Jaynes *et al.* 1987, Hightower *et al.* 1994) and cecropin B (Jaynes *et al.* 1993). In addition, we extended these observations to include the activity of cecropin P1 against *Erwinia*, and also established the potential of magainins as control agents against plant pathogens. In all our comparisons of peptide activity, the cecropins displayed greater activity than magainins (Table 2, Fig. 1). This is consistent with the results of Wade *et al.* (1990), who reported cecropins to have an order of magnitude higher activity than magainins on a molar basis. In contrast to the reported activity of caerin 1.1 against a wide range of microbes (Stone *et al.* 1992), we were unable to detect any significant activity against *Streptomyces* and *Erwinia* at the concentrations used in this study.

At the peptide concentration used, complete inhibition of the test pathogens was only found with treatments of cecropins against *Erwinia*. In all tests with *Streptomyces* sp. at least a few bacterial colo-

nies were observed to survive *in vitro* peptide treatments. Further tests on these rare bacteria are underway to determine whether they result from the development of resistance or are simply due to insufficient peptide within each reaction tube thus allowing a proportion of cells to escape lysis prior to dilution plating. If the former is true then the long term prospects of utilising a single peptide for disease control is questionable. However, it is possible to incorporate into the same plant genes encoding several distinct types of antimicrobial factors, which may act in tandem to reduce the risk of resistance breaking mutations. In fact, such a multifactor system is present in the organisms from which these peptides originated (Hultmark *et al.* 1980). Furthermore, the simultaneous presence of more than one peptide may result in a synergy of activity, as demonstrated in this study by the combined treatments of cecropin A and magainin 2. Other synergistic reactions between cecropins and the larger but less potent antibacterial polypeptides lysozyme and attacin have also been demonstrated (Jaynes *et al.* 1993, Boman *et al.* 1985).

The construction of vectors with chimeric genes may necessitate minor alterations in peptide sequence to obtain proper expression of antimicrobial peptide in plants. Minor changes to peptide sequence have been shown to not adversely affect the antimicrobial activity, in fact specific changes can result in greatly enhanced activity (Chen *et al.* 1988, Jaynes *et al.* 1993). Some changes, like the amidification of magainin 2 amide peptide, can not be encoded within a DNA construct. For this reason further tests with this peptide were not performed.

Pathogenic *Streptomyces* sp. are known to possess a high degree of variability (Tashiro *et al.* 1990, Healy & Lambert 1991, Doering-Saad *et al.* 1992 Faucher *et al.* 1992). Therefore the isolates chosen for this study were from a diverse range of distinct strains. They included representatives of both currently classified species implicated with common scab disease, and differed in geographical source and virulence (isolate 25 was less aggressive than the other isolates). It is noteworthy that all isolates reacted similarly with no marked differences in their sensitivities to the peptide treatments.

Like other bacterial pathogens, *Streptomyces* sp. mainly colonise the intercellular spaces of susceptible plants during infection (Agrios 1978). Cellular disruption occurs in advance of the pathogen's growth through the production of phytotoxins (King *et al.* 1991) thus allowing the pathogen to grow saprophytically on released cell contents. Therefore, it is important for any antimicrobial agent to accumulate within these intercellular spaces. With respect to gene technology, this is possible by fusing the antimicrobial gene downstream of an appropriate plant signal sequence. Such translation fusions to signal peptides are known to facilitate the export of peptides from plant cells (Hippe *et al.* 1989, Sijmons *et al.* 1990).

Streptomyces sp. are useful target organisms for this approach to disease control. Unlike many bacterial pathogens, the disease progresses relatively slowly and does not extensively degrade host plant tissues (Agrios, 1978). The plant would have opportunity to produce and accumulate sufficient peptide to counter any slow developing infection.

This approach involving transfer and expression of genes encoding cecropin and magainin peptides to potatoes could be a powerful technique for development of resistance to common scab, black leg and tuber soft rot disease. Given the reported activity of these peptides against a wide range of other bacteria, fungi, and protozoa (Zasloff *et al.* 1988, Jaynes *et al.* 1988, Wade *et al.* 1990), there may also be additional benefits for the control of a wide range of other pests and diseases.

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